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A method for fast and simple detection of major diarrhoeagenic *Escherichia coli* in the routine diagnostic laboratory

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ABSTRACT

A multiplex PCR was developed for the detection of the following genes characteristic of diarrhoeagenic *Escherichia coli* (DEC): verocytotoxins 1 (*vtx1*) and 2 (*vtx2*), characteristic of verocytotoxin-producing *E. coli* (VTEC); intimin (*eae*), found in enteropathogenic *E. coli* (EPEC), attaching and effacing *E. coli* and VTEC; heat-stable enterotoxin (*estA*) and heat-labile enterotoxin (*eltA*), characteristic of enterotoxigenic *E. coli* (ETEC); and invasive plasmid antigen (*ipaH*), characteristic of enteroinvasive *E. coli* (EIEC) and *Shigella* spp. The method allowed the simultaneous identification of all six genes in one reaction, and included a 16S rDNA internal PCR control. When applied to pure cultures from a reference strain collection, all virulence genes in 124 different DEC strains and 15 *Shigella* spp. were identified correctly, and there were no cross-reactions with 13 non-*E. coli* species. The detection limit of the method was 10^2–10^3 DEC CFU/PCR in the presence of 10^6 non-target cells. When the multiplex PCR was tested with colonies from plate cultures of clinical stool samples, it was a faster, more sensitive, less expensive and less laborious diagnostic procedure than DNA hybridisation. When used with DNA purified from spiked stool samples (by two different commercial kits), the method had a detection limit of 10^6 CFU/mL stool sample.

Keywords Detection, diarrhoeagenic *Escherichia coli*, EIEC, EPEC, ETEC, multiplex PCR

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INTRODUCTION

Diarrhoeagenic *Escherichia coli* (DEC) is a significant contributor to diarrhoeal disease throughout the world [1–3]. The symptoms of DEC illness range from self-limiting watery diarrhoea to life-threatening illness, with children, the elderly, those suffering from malnutrition, and immunocompromised individuals being at higher risk of developing serious complications. Based on the pathogenesis, clinical manifestations and presence of specific virulence factors, the major DEC groups include [4]: (i) verocytotoxin-producing *E. coli* (VTEC); (ii) enteropathogenic *E. coli* (EPEC); (iii) enterotoxigenic *E. coli* (ETEC); (iv) enteroinvasive *E. coli* (EIEC); and (v) enteroaggregative *E. coli* (EAggEC).

VTEC strains produce verocytotoxins VT1 and/or VT2, encoded by the *vtx1* and *vtx2* genes, respectively. VTEC strains account for the most severe clinical manifestations among the DEC strains, including gastroenteritis and bloody diarrhoea, sometimes leading to haemolytic uraemic syndrome. EPEC strains cause characteristic attaching and effacing lesions in the small intestine, which are associated with the virulence factor intimin, encoded by the *eae* gene. The *eae* gene may also be present in VTEC strains, and is always present in attaching and effacing *E. coli* strains, which are less virulent or non-virulent. ETEC strains cause diarrhoea because they produce one or both of two enterotoxins, heat-stable enterotoxin and heat-labile enterotoxin, encoded by the *est* and *elt* genes, respectively. EIEC strains are related closely to *Shigella* spp. with respect to both phylogeny and pathogenesis. Characteristic of the

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invasive phenotype of both EIEC strains and *Shigella* spp. is the *ipaH* gene, which is present in several copies on both the chromosome and the invasive plasmid (pInv). EAggEC strains are probably a frequent cause of diarrhoea in both the industrialised and the developing world, and several putative virulence factors have been found in EAggEC strains. However, all these virulence factors are also encountered in other *E. coli* groups, and none occur in all EAggEC strains. Therefore, EAggEC strains cannot be identified simply by detection of specific virulence factors or genes. Cell adherence assays comprise the reference standard method, and are the only truly reliable methods described to date for identifying these strains. A large, diverse group of *E. coli* strains, characterised by their diffuse adherence to cells, has also been described. The pathogenic potential of this group still needs to be confirmed.

Serotyping and biochemistry have been widely applied in the diagnosis of gastrointestinal pathogens, but cannot be used for conclusively identifying DEC groups. Therefore, identification of the characteristic virulence genes is an obvious choice for DEC diagnostics. Since the discovery of the virulence genes, DNA hybridisation has been a successful method for the detection of DEC [5–11], but PCR has become the preferred diagnostic tool, because of the shorter time required for analysis and the simpler and less expensive experimental procedure. PCR analyses for identification of DEC groups have been designed that detect one or a few genes per reaction [10,12–24]. However, PCRs are now being designed to detect multiple genes in the same reaction, thereby further reducing the cost and time required for the experimental procedure [22,25–33].

The aim of the present study was to develop a PCR method for routine diagnostic identification of DEC infections caused by VTEC, EPEC, ETEC and/or EIEC by simultaneous and specific detection of the six virulence genes (*eltA*, *estA*, *vtx1*, *vtx2*, *eae* and *ipaH*). As new gene variants are constantly being submitted to GenBank, the most complete and updated sequence data were used to design primers for the detection of individual genes. For routine diagnostic purposes, the assay also included the UNG-dUTP carryover prevention system, together with an additional primer pair targeting a universal 16S rDNA sequence as a positive internal PCR control. This study describes the method design and its validation using DNA obtained from primary stool cultures in comparison with the reference standard DNA hybridisation technique. The assay's capabilities were also tested with spiked stool specimens in order to further reduce the analysis time by circumventing the initial culture step.

MATERIALS AND METHODS

Strains and DNA preparations

All strains used in this study were obtained from the International *Escherichia* and *Klebsiella* Centre (WHO), Statens Serum Institut, Copenhagen, Denmark. Before analysis, selected strains were grown on agar plates overnight at 37°C. One colony was transferred to 100 μL of Chelex 100 (Bio-Rad, Hercules, CA, USA) 10% w/v in 10 mM Tris-HCl, 1 mM EDTA, pH 8, boiled for 5 min, and centrifuged briefly. The resulting supernatant (5 μL) was used directly as a DNA template in PCRs. Liquid cultures for the sensitivity and spiking experiments were prepared from single colonies inoculated and grown in Luria broth overnight at 37°C. Cell densities of the liquid cultures were determined by counting colonies from ten-fold dilutions on three replicate plates. For the sensitivity experiment with pure cultures, 5 μL of liquid culture was diluted ten-fold in Chelex 100 in 10 mM Tris-HCl, 1 mM EDTA, pH 8, and treated as above. These dilution series resulted in 10^{7}–10^{-1} and 0 DEC CFU/PCR. Four dilution series were constructed: (i) an ETEC strain (*estA* and *eltA*) and an EIEC strain (*ipaH*); (ii) a VTEC strain (*vtx1*, *vtx2* and *eae*); (iii) an ETEC strain (*estA* and *eltA*) and an EIEC strain (*ipaH*), combined with a non-pathogenic *E. coli* strain at 10^{6} CFU/PCR in each dilution; and (iv) a VTEC strain (*vtx1*, *vtx2* and *eae*) combined with a non-pathogenic *E. coli* strain at 10^{6} CFU/PCR in each dilution.

Culture and DNA preparation from stool

Stool samples (*c.* 0.1 g) were stirred in 2 mL of sterile buffered saline (80 mM NaCl, 50 mM Na_2HPO_4, 10 mM KH_2PO_4, pH 7.38). An aliquot (*c.* 10 μL) of this suspension was plated on an SSI enteric medium plate (SSI Diagnostica, Hillerød, Denmark [34]) and grown overnight at 37°C. *E. coli* strains may grow with different colony morphologies on this medium. From these plates, two examples of each morphologically different *E. coli*-like colony type were selected and pooled, and DNA was extracted as described above, except that the supernatant was diluted ten-fold in water before PCR analysis. A sweep of culture material from an area on the plate with semi-confluent or confluent growth was also tested, and this material was treated as described above. Colonies and areas of confluent growth were also transferred to individual spots on a nylon membrane for subsequent analysis by DNA hybridisation.

Two bloody and two non-bloody stool samples (all negative for gastrointestinal pathogens) were chosen for the spiking experiments. Liquid cultures of either a VTEC strain (containing *eae*, *vtx1* and *vtx2*), or an ETEC strain (containing *eltA* and *estA*) combined with an EIEC strain (containing *ipaH*), were added to the stools, resulting in final concentrations in stools of

10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 0 DEC CFU/mL. Template DNA for the PCR was extracted from stools using two commercially available DNA extraction systems, the automatic KingFisher mL system (Thermo Labsystems, Vantaa, Finland) and the manual QIAamp DNA Stool Kit (Qiagen, Hilden, Germany), according to the manufacturers' instructions. Ten-microlitre samples of the spiked stools were also grown on SSI enteric medium plates as described above. DNA was extracted from morphologically different colonies by the simple boiling procedure, and was then analysed by PCR as described above.

DNA hybridisation

DNA hybridisation was performed with probes for *vtx1* [35], *vtx2* [11], *eae* [36], *elt* and *estA-h*, and *estA-p* [6–8] and *ipaH* [37]. Cell material from the SSI enteric medium plate was allowed to grow on the nylon membrane positioned on top of an agar plate. The colonies were lysed, denatured and neutralised using standard conditions and then hybridised with digoxigenin-labelled probes under stringent conditions according to the manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany).

Multiplex PCR

PCRs were performed in a total reaction volume of 25 μL containing 1 × PCR buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM $(NH_4)_2SO_4$, pH 8.3), 2.6 mM $MgCl_2$, 260 μM each of dATP, dCTP and dGTP, 520 μM dUTP, 0.15 U of UNG (Applied Biosystems, Foster City, CA, USA), 1.25 U of *Taq* polymerase (FastStart; Roche Diagnostics), and the 16 primers shown in Table 1 in the listed concentrations (DEC Primer Mix; SSI Diagnostica, Hillerød, Denmark). These included a primer pair for the 16S rDNA gene as a positive internal PCR control. Template volumes were 5 μL when PCRs were performed with bacterial cultures and stool samples extracted with the QIAamp DNA Stool Kit, and 1 μL when stool samples were extracted with the KingFisher mL system. Amplification conditions comprised 94°C for 6 min, followed by 35 cycles of 94°C for 50 s, 57°C for 40 s and 72°C for 50 s, and finally 72°C for 3 min. Amplicons were analysed by electrophoresis on agarose 1.5% w/v gels using standard conditions, followed by staining with ethidium bromide.

RESULTS

Assay specificity tested with reference strains

The assay specificity was tested with a strain collection that included three non-pathogenic *E. coli* strains, 15 *Shigella* spp. and 124 different DEC strains, representing a variety of different serotypes and the following genetic subtypes: *vtx1*, *vtx1c*, *vtx2*, *vtx2c*, *vtx2d*, *vtx2e* (according to [38]), *eae-α*, *eae-β*, *eae-γ*, *eae-δ*, *eae-ϵ*, *eae-ζ* and *eae-θ*. Primer sequences, gene targets and amplicon sizes are listed in Table 1. The six virulence genes were identified correctly in all strains (Table 2). As expected, *E. coli* strains characterised by their diffuse adherence to cells, EAggEC strains (containing the EAST1 toxin) and non-pathogenic *E. coli* strains did not produce any PCR products, except the 16S rDNA band. Fig. 1 shows the results obtained with 13 selected reference strains. Amplicons were identified by comparison with size markers; successful amplification of the 16S rDNA band (1062 bp) in each PCR was required for a valid result.

The specificity was also tested using 13 non-*E. coli* intestinal colonising species grown on SSI enteric medium, comprising *Salmonella* Enteritidis, *Salmonella* Paratyphi A, *Salmonella* Typhimurium, *Vibrio cholera* non-O1, *Aeromonas caviae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Plesiomonas shigelloides*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Salmonella* Cholerasuis and *Yersinia enterocolitica*. None of these bacteria gave rise to PCR products, except the 16S rDNA amplicon.

Table 1. Gene targets, primer sequences, primer concentrations and amplicon sizes for the multiplex PCR

Primer	Gene target	Virulence factor/gene	Sequence (5′-)	Final concentration (μM)	Amplicon size (bp)
StFh	Human *estA*	STIh	TTTCGCTCAGGATGCTAAACCAG	0.4	151
StRh			CAGGATTACAACACAATTCACAGCAGTA	0.4	
StFp	Porcine *estA*	STIp	CTTTCCCCTCTTTTAGTCAGTCAACTG	0.4	160
StRp			CAGGATTACAACAAAGTTCACAGCAG	0.4	
PS3	*vtx1*	VT1	GTTTGCAGTTGATGTCAGAGGGA	0.25	260
PS4			CAACGAATGGCGATTTATCTGC	0.25	
PS5	*eae*	Intimin	GGYCAGCGTTTTTTCCTTCCTG	0.15	377
PS6			TCGTCACCARAGGAATCGGAG	0.15	
PS7	*vtx2*	VT2	GCCTGTCGCCAGTTATCTGACA	0.5	420
PS8			GGAATGCAAATCAGTCGTCACTC	0.5	
PS9	*eltA*	LTI	AAACCGGCTTTGTCAGATATGATGA	0.45	479
PS10			TGTGCTCAGATTCTGGGTCTCCT	0.45	
PS11	*ipaH*	IpaH	TTGACCGCCTTTCCGATACC	0.1	647
PS12			ATCCGCATCACCGCTCAGAC	0.1	
PS13	16S rDNA	16S rDNA	GGAGGCAGCAGTGGGGAATA	0.25	1062
PS14			TGACGGGCGGTGTGTACAAG	0.25	

R = A or G; Y = C or T.

Table 2. Results obtained using the multiplex PCR method for 142 reference strains from the International *Escherichia* and *Klebsiella* Centre (WHO)

E. coli group/ *Shigella*	No. of strains	Serotype (no. of each serotype)	Virulence profile						
			estA	*vtx1*	*eae*	*vtx2*	*eltA*	*ipaH*	Negative
A/EEC	23	Orough:H8 (1), Orough:H33 (1), O4,O123:H– (1), O35,O135:H11 (1), O51:H49 (1), O86:H8 (2), O103:H2 (1), O111:H38 (1), O114:H49 (1), O116:H+ (1), O118:H8 (1), O121:H19 (1), O125ab:H5 (1), O126:H6 (1), O129: H11 (1), 132:H34 (1), O145:H– (3), O145:H34 (1), O157:H7 (1), O177:H25 (1)			+				
EPEC	9	O26:H– (3), O55:H7 (1), O111:H– (1), O111:H9 (1), O114:H– (1), O127:H– (1), O142:H34 (1)			+				
EIEC	13	O+:H– (2), O28ac:H– (2), O64:H– (2), O121:H– (1), O124:H30 (1), O143:H– (2), O144:H– (1), O172:H– (1), O173:H– (1)						+	
Shigella	12	*S. sonnei* (3), *S. flexneri* 6 (2), *S. flexneri* 1b (2), *S. flexneri* 2a (1), *S. boydii* 1-7 (1), *S. dysenteriae* 2-7 (1), *Shigella* non-agglutinable (2)						+	
Shigella	3	*S. dysenteriae* Type 1		+				+	
DAEC	4	O15:H– (1), O21:K–:H11 (1), O21:10 (1), O36:H4 (1)							+
EAggEC	8	O24:H+ (1), O92:H+ (1), O92:H33 (1), O103:H+ (1), O107:H+ (1), O113:H– (1), O150:H28 (1), O153:H2 (1)							+
Non-pathogenic	3	O9:K+:H4 (1), OR:K?:H25 (1), OR:H48 (1)							+
ETEC	8	Orough:H– (1), O6:H16 (1), O8:H9 (1), O17:Hrough (1), O39:H12 (1), O78:K–:H11 (1), O128ac:H+ (1), O148:H28 (1)	+				+		
ETEC	5	O8:K+:H9 (1), O27:K?:H7 (1), O27:K–:H20 (1), O115:K?:H5 (1), O148:H28 (1)	+						
ETEC	7	Orough:H– (1), O8:K+:H9 (1), O8:H9 (1), O25:K+:H– (1), O56:H– (1), O167:Hrough (1), O169:H– (1)					+		
VTEC	9	O26:H– (1), O26:H11 (1), O157:H– (5), O157:H7 (2)		+	+	+			
VTEC	19	O26:H11 (5), O103:Hrough (1), O103:H2 (4), O145:H+ (3), O145:H28 (2), O157:H– (1), O157:K:H– (1), O157:H7 (1), O157:K–:H7 (1)		+	+				
VTEC	19	O26:H– (1), O26:H11 (5), O103:H2 (3), O145 (1), O145:H– (5), O157:H– (1), O157:H7 (4)			+	+			
Total	142								

A/EEC, attaching and effacing *Escherichia coli*; EPEC, enteropathogenic *E. coli*; EIEC, enteroinvasive *E. coli*; DAEC, diffuse adherence to cells *E. coli*; EAggEC, enteroaggregative *E. coli*; ETEC, enterotoxigenic *E. coli*; VTEC, verocytotoxin-producing *E. coli*.

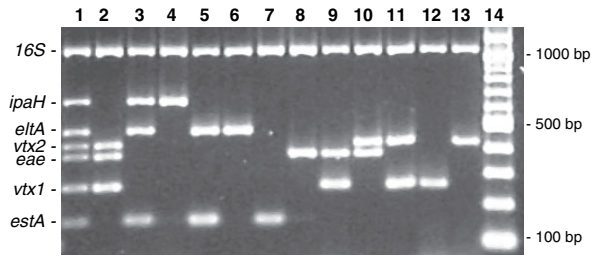

Fig. 1. PCR results obtained with 13 selected reference strains. Lanes: 1 (all genes), ETEC (*estA* and *eltA*), VTEC (*vtx1*, *vtx2* and *eae*) and EIEC (*ipaH*); 2, VTEC (*vtx1*, *vtx2* and *eae*); 3, EIEC (*ipaH*) and ETEC (*estA* and *eltA*); 4, EIEC (*ipaH*); 5, ETEC (*estA* and *eltA*); 6, ETEC (*eltA*); 7, ETEC (*estA*); 8, EPEC (*eae*); 9, VTEC (*vtx1* and *eae*); 10, VTEC (*vtx2* and *eae*); 11, VTEC (*vtx1* and *vtx2*); 12, VTEC (*vtx1*); 13, VTEC (*vtx2*); and 14, 100-bp DNA ladder.

Assay specificity with clinical isolates in comparison with DNA hybridisation

The multiplex PCR method was compared with a DNA hybridisation technique for 500 stool samples originating from patients with diarrhoea (Table 3). Stool samples were first cultured on SSI enteric medium plates, from which colonies and areas of confluent growth were selected and tested by DNA hybridisation and multiplex PCR. When the analysis was based on colonies, there was agreement between the two methods for 495 samples (466 negative and 29 positive). An additional five samples were positive only by PCR. Depending on the amount of stool material and the inoculation density, each plate might have a significant amount of confluent growth,

Table 3. Comparison of results obtained using multiplex PCR and DNA hybridisation for the identification of colonies grown from 500 clinical stool samples on SSI enteric medium plates

Gene(s)	ID by both methods with colonies	ID only by PCR with colonies	ID only by PCR with confluent growth[a]
Negative	466		
eltA	3		
eae	20	2	7 (3)
vtx1	1	1	1 (0)
vtx1 + eae	1		
vtx2	1	1	
ipaH	3	1	3 (2)
Total positive	29	5	11 (5)

[a]No. of samples from which single positive colonies were subsequently obtained is shown in parentheses.

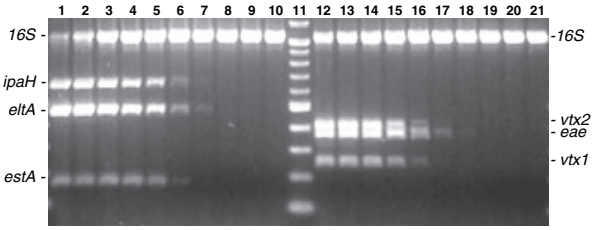

Fig. 2. Sensitivity of the multiplex PCR with pure cultures. DEC reference strains were serially diluted ten-fold and mixed with a non-pathogenic *Escherichia coli* strain, resulting in each dilution containing 10^6 non-pathogenic *E. coli*, and 10^7–10^{-1} and 0 DEC in lanes 1–10 and 12–21. Lanes: 1–10, reference strain ETEC (*estA* and *eltA*) and EIEC (*ipaH*); 11, 100-bp DNA ladder; 12–21, reference strain VTEC (*vtx1*, *vtx2* and *eae*). When strains containing *estA*, *eltA* and *ipaH* were diluted, positive signals were obtained until a concentration of 100 CFU/PCR (lane 6) was reached. When a strain containing *vtx1*, *vtx2* and *eae* was diluted, positive signals were obtained until a concentration of 1000 CFU/PCR (lane 16) was reached.

containing cell material originating from thousands of different colonies. In order to test the sensitivity of the multiplex PCR in practice, a sweep of the confluent growth on each plate was therefore tested by PCR and DNA hybridisation. PCR analysis of the sweeps was positive for all samples that were also positive using the single colony procedure. In addition, 11 previously negative samples were positive following PCR analysis of the sweeps. None of these samples was positive following DNA hybridisation with the extracted sweep material. Single colonies were isolated from five of the 11 PCR-positive samples.

Sensitivity with pure cultures

The sensitivity of the multiplex PCR was tested by analysing ten-fold serial dilutions of DEC reference strains mixed with non-pathogenic *E. coli* (Fig. 2). The detection limit for the *ipaH*, *eltA* and *estA* genes was 100 CFU/PCR in the presence of 10^6 non-target cells (Fig. 2, lanes 1–10), while the *vtx1*, *vtx2* and *eae* genes were detectable at 1000 CFU/PCR in the presence of 10^6 non-target cells (Fig. 2, lanes 12–21). When the analysis was performed in the absence of the non-target cells, a ten-fold higher sensitivity was found for all genes (data not shown).

Analysis of spiked stool samples

Stools were spiked with ten-fold serial dilutions of a VTEC strain, or with an ETEC and an EIEC strain. For each strain combination, two bloody and two non-bloody stool samples were extracted using either the KingFisher mL system or the QIAamp DNA Stool Kit, followed by analysis with the multiplex PCR. Each virulence gene was detected at a concentration of 10^6 CFU/mL of extracted stool using both extraction procedures, except one bloody stool extracted using the KingFisher mL system, from which no DNA amplicons were obtained. The spiked stools were also cultured on SSI enteric medium, followed by PCR with selected colonies, which resulted in a detection limit of 10^5 CFU/mL of stool.

DISCUSSION

This study describes the development of a multiplex PCR method for the detection of the most important DEC groups, namely ETEC, EPEC, VTEC and EIEC. Each pathogen is identified by the presence and combination of six virulence genes: *eltA*, *estA*, *vtx1*, *vtx2*, *eae* and *ipaH*. Primers directed towards all six virulence genes, as well as a 16S rDNA gene serving as a positive PCR control, were included in the multiplex assay. The method was 100% specific when tested with a representative reference strain collection that included 127 different *E. coli* strains and 15 *Shigella* spp. Also, no cross-reactions were observed with 13 non-*E. coli* gastrointestinal pathogens. Therefore, the method appears to be well-suited for detection of DEC in culture material. However, genetic assays do not allow a distinction between EPEC and attaching and effacing *E. coli*, as the virulence factor(s) responsible for

this differentiation have not been identified to date; currently, this can only be done by serotyping [39]. Also, *ipaH*- and *vtx1*-positive strains must be further identified by biochemical tests, as these genes may also be present in *Shigella* spp. [4]. Finally, because of the diffuse virulence profile of EAggEC, these strains were not included in the development of this PCR method.

As both human *estA* and porcine *estA* have been found in humans [4], both of these variants were included in the PCR. Separate primer pairs were designed for each variant because of sequence diversity, but the products (151 and 160 bp) were indistinguishable when analysed by the present agarose gel electrophoresis. Primers were not designed to detect the gene encoding STII/STb (*estB*) (accession nos M35729 and AY028790), as these variants are rarely found in humans [4,40]. The primers for *estA* did not align with *Y. enterocolitica* heat-stable enterotoxin (D63578), *V. cholerae* non-O1 heat-stable enterotoxin (accession no. M97591) or EAggEC heat-stable enterotoxin 1, EAST1 (accession no. AB042005), although they share some sequence similarities. Primers towards the heat-labile enterotoxin were designed to match human and porcine *eltA* only, as *eltB* has not been associated with human disease [4]. The *eltA* primers did not match the related *V. cholerae* cholera enterotoxin gene *ctxA* (accession no. AF452584). Because of sequence conservation between EIEC and *Shigella* spp., primers for the *ipaH* gene were designed to match all variants from both species, and primers for *vtx1* matched all variants within subtypes *vtx1* and *vtx1c* from VTEC and *Shigella dysenteriae* type I. Primers targeted to *vtx2* were designed to match all variants, except subtype *vtx2f* (accession no. AJ270998), which was too divergent to be included and has little human relevance [41,42]. However, *vtx2* genes with a high degree of homology have been described previously in *Enterobacter cloacae* (accession no. Z50754) [43] and *C. freundii* (accession no. X67514) [44], and these genes are also amplified by the present method. Primers for the amplification of *eae* contained one degenerate nucleotide each, and were designed to match all subtype variants.

The sensitivity analysis showed that each pathogen could be detected by the multiplex PCR at a level of 10^1–10^2 CFU/PCR when pure cultures were analysed. This level of sensitivity is in the same range as that found by others when analysing pure cultures [20,26,32], and is an acceptable sensitivity for a multiplex reaction analysed subsequently by agarose gel electrophoresis and ethidium bromide staining. However, when 10^6 non-target cells were present, the sensitivity of detection for each locus dropped ten-fold. This finding has also been reported previously [45], and may be caused by the reduced possibility of physical contact among the PCR reagents. Information concerning sensitivity in the presence of non-target cells is especially important in the context of analysing mixed cultures, such as stool samples. In such instances, the virulence loci of interest may be highly diluted in relation to non-target DNA. The present sensitivity analysis with pure cultures showed that each pathogen could be detected at a level of 10^2–10^3 CFU/PCR in the presence of 10^6 non-target cells. Thus, positive samples can be identified correctly if the positive bacterial cell material accounts for 1/1000 of the total cell material.

The PCR method was compared with a colony dot-blot DNA hybridisation technique for the analysis of stool cultures, as the latter method was the preferred routine diagnostic method at the Statens Serum Institut between 1997 and 2003. Both methods gave concordant results for 495 of 500 samples, while five samples were positive only by PCR. Ideally, these five samples should have been tested by other methods to exclude false-positive results. When confluent growth on the plates was subjected to PCR and DNA hybridisation, 11 previously negative samples were positive only by PCR. However, only five of these 11 samples could be re-isolated as single positive colonies when the confluent growth was streaked out on a new plate, probably because relatively few positive cells are hidden in the confluent growth. No studies were performed to determine the detection limit of DNA hybridisation, but these observations probably reflect a greater sensitivity of the PCR analysis compared with DNA hybridisation.

To reduce repetitive manual work and possible cross-contamination, the present method was performed using eight-channel pipettes and microtitre plates in all steps, including sample collection and preparation, PCR and gel loading. As generally recommended for routine diagnostic PCR, the various steps were performed in separate rooms with unidirectional

sample flow [46,47]. To reduce possible contamination, UNG-dUTP was used, and all pre-PCR and PCR work areas were illuminated by UV light at night. The 16S rDNA positive control primers were designed to detect all Gram-negative bacteria, and hence PCR analyses of negative samples can be validated, even if they contain no *E. coli*.

The multiplex PCR method was used in conjunction with DNA purified from spiked stools using two different commercial kits. Both methods are based on DNA liberation in lysis buffer, and subsequent DNA purification following binding to immobilised silica. With the exception of one bloody stool extracted by the KingFisher mL system, all spiked stools were detected correctly by both methods at a level of $\geq 10^6$ CFU/mL of stool. Compared to the detection limit for pure cultures plus non-target cells, both commercial extraction methods performed well with respect to recovery of DNA and removal of PCR inhibitors known to be present in blood and faecal material [48–52]. The relative reduced sensitivity for stool samples could be the result of DNA loss during the extraction procedure, and/or $>10^6$ CFU of non-target cells in the original stools. The single bloody stool sample that could not be amplified by PCR might have exceeded the PCR-inhibitor-extracting capacity of the KingFisher mL method. This unsuccessful amplification was recognised by the absence of the 16S rDNA control band, which highlights the need for a second PCR or another type of analysis.

Previous studies have reported up to 100-fold higher sensitivities when DNA was extracted from spiked stool samples [53–56], possibly caused by differences in extraction methods and/or the larger number of gene targets included in the present multiplex PCR. When the spiked stools were investigated following growth on SSI enteric medium plates, with morphologically different colonies being selected for the multiplex PCR analysis, a sensitivity limit of 10^5 CFU/mL stool was obtained. Previous studies have reported sensitivities as low as 100 CFU/g of stool following the use of pre-PCR enrichment steps [28,29], but this has the disadvantage of a more lengthy procedure before the isolation of single positive colonies. Whether or not clinical samples are cultured before PCR, the approach described in the present study appears to be valuable for routine diagnostic identification of DEC.

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